

Sperm phenotypic plasticity in a cichlid: a territorial male's counterstrategy to spawning takeover

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Studies of sperm competition in species with alternative reproductive tactics (ARTs) often pay attention to the differences in investments in sperm between sneakers facing a higher sperm competition risk and bourgeois males facing a lower risk. Here, we examined within-tactic as well as among-tactic variations in sperm investments in the Lake Tanganyika shell-brooding cichlid *Telmatochromis vittatus*, a species with 2 types of parasitic tactics. Territorial male *T. vittatus* suffer reproductive parasitism by both smaller sneaker males and larger pirate males ("pirates" take over the spawning event during which territorial males perform sneaking as a counterstrategy). We hypothesized that both territorial males living under the risk of pirates and sneakers face increased risk of sperm competition and therefore should produce high-quality sperm compared with both territorial males that experienced no piracy risk and pirates. As expected, field studies showed that the former 2 males produced longer lived sperm than the other males. Aquarium experiments demonstrated that a visual stimulus of a pirate was enough to induce an increase in sperm longevity in territorial males compared with when no such stimulus was given. These results indicate that territorial male *T. vittatus* can plastically adjust at least one sperm quality trait in response to piracy risk. Moreover, long-term monitoring of males in the field showed that small territorial males grow into large territorial males and finally into pirates, so ARTs are not fixed over life. Accordingly, male *T. vittatus* appear to ontogenetically change their sperm longevity in response to size-dependent sperm competition risks. **Key words:** preoviposition ejaculate, reproductive parasitism, sperm competition, sperm quality traits. [*Behav Ecol* 21:1293–1300 (2010)]

Sperm competition, a form of sexual selection after insemination, is widespread in a variety of animal taxa and has a strong evolutionary force that leads to adaptations related to male and female reproductive anatomies and behaviors (Birkhead and Møller 1998; Simmons 2001; Birkhead and Pizzari 2002). Male competitive success in sperm competition can be, for instance, affected by the relative numbers of sperm among competitors, resulting in the increased investment in testes under high levels of sperm competition (Parker 1990; Wedell et al. 2002, for review). Comparative studies across species have indeed found that species experiencing a higher risk of sperm competition invest more in testes that produce more sperm (e.g., Harcourt et al. 1981; Møller 1991; Gage 1994; Stockley et al. 1997; Byrne et al. 2002).

Recently, it has been recognized that sperm quality traits, such as sperm size, longevity, and swimming speed, play important roles in determining male fertilization success both under conditions of sperm competition and noncompetitive contexts (Froman et al. 1999; Levitan 2000; Kupriyanova and Havenhand 2002; Gage et al. 2004; Casselman et al. 2006; Liljedal et al. 2008; Rudolfsen, Figenschou, et al. 2008; Snook 2005). Comparative studies have often detected a significant covariation between sperm quality traits and sperm competition

risks (e.g., Gomendio and Roldan 1991; Stockley et al. 1997; Balshine et al. 2001; Fitzpatrick et al. 2009; Kleven et al. 2009). Another informative approach is to examine at the within-species level whether and how males respond to sperm competition risk. In this respect, animal species where individual males follow alternative reproductive tactics (ARTs) are ideal because it is expected that there are variations in sperm competition risks between tactics within a species, and males are expected to invest in sperm numbers and quality accordingly. In fish with ARTs, sperm competition typically occurs when reproductively parasitic males steal fertilization opportunities from bourgeois males that attempt to monopolize fertilization by defending mates during mating (Petersen and Warner 1998; Taborsky 1998). Consequently, parasitic males are always subject to sperm competition, whereas bourgeois males may often experience lower or no risk of sperm competition due to effective territoriality (Alonzo and Warner 2000; Sato et al. 2004; Scaggiante et al. 2005). Mate guarding by bourgeois males often makes parasitic males fail to ejaculate close to the ova or force parasitic males to time their sperm release suboptimally (Kano 1996; Stoltz and Neff 2006). Thus, parasitic males often play a disfavored role in sperm competition. Many empirical studies have shown that parasitic males produce more and higher quality sperm compared with bourgeois males to counterbalance any disadvantage they might have during spawning (e.g., Gage et al. 1995; Simmons et al. 1999; Leach and Montgomerie 2000; Uglem et al. 2001; Vladić and Järvi 2001; Neff et al. 2003; Burness et al. 2004; Rudolfsen et al. 2006; Serrano et al. 2006; Fitzpatrick et al. 2007; Locatello et al. 2007).

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Sperm competition risk may not only vary between male tactics but also within tactics. For example, the likelihood of reproductive parasitism is often not uniformly distributed among the nests in the wild populations of fish species (Goldschmidt et al. 1992; Picciulin et al. 2004; Ota and Kohda 2006b). This unequal distribution might come about due to territorial males experiencing different levels of sperm competition risk, but whether territorial males respond to this variation has seldom been examined, especially in natural habitats. Here, we examined 1) whether males of different ARTs differ in their sperm quality (longevity, flagellum length, and swimming speed) and sperm quantity (using residual gonad mass (GM) of the regression between GM vs. soma mass (SM) as a proxy) and 2) whether bourgeois males under experimentally increased risk of sperm competition invest more in sperm quality and quantity (i.e., residual GM) than territorial males facing no such risk. As a model species, we use the Lake Tanganyika cichlid *Telmatochromis vittatus*, in which males express 4 types of ARTs: territorial males, 2 types of reproductively parasitic males, and nonparasitic satellite males (Ota and Kohda 2006a).

Territorial male *T. vittatus* court and spawn with females in his nest, which consists of about 80 empty snail shells (these nests are constructed by male *Lamprologus callipterus*, male *T. vittatus* coinhabit these nests, see Ota and Kohda 2006a). During several hours of a single spawning event, a female stays inside her shell, where she deposits 20–100 eggs one by one on the inner wall. Territorial males position their genital papilla over the shell entrance for a few seconds and ejaculate into the shell whenever an egg is laid. Territorial males are occasionally reproductively parasitized by 2 types of males: small males called “sneakers” and large males called “pirates” (Ota and Kohda 2006a). Sneakers, which are much smaller than the territorial male, dart to the shell containing the spawning female and quickly ejaculate over the entrance. Their sperm always has to compete for fertilization with the sperm from the territorial male and potentially also with sperm from additional sneakers and a pirate (Ota and Kohda 2006a, 2006b). Territorial males may repel sneakers successfully by intense mate guarding and actually only in 14% of all spawning events and 2.5% of all potential sneaking attempts, sneakers are successful in releasing sperm at least once (Ota and Kohda 2006b; Ota 2007). Therefore, territorial males are under limited sperm competition risk from sneakers. In contrast, territorial males are under a very high sperm competition risk from pirates. This is because pirates, which are substantially larger than the targeted territorial males and do not occupy their own nests, take over the spawning event from the territorial males, essentially usurping the spawning until the spawning event is completed. Pirates ejaculate at the shell entrance like territorial males. When territorial males are pirated, they switch their behavior and attempt to sneak on the pirate inside their own nests (Ota 2007).

Due to the spatial distribution of territorial males and pirates and because pirates can only takeover spawning of smaller sized territorial males, individual territorial males differ widely in their risk of experiencing pirating (Ota and Kohda 2006a). In the first part of this paper, we examine sperm traits of wild-caught males of the 3 ARTs, and we use the variation in the risk of pirating to divide the territorial males into 2 groups: those territorial males which are likely to face sperm competition from pirates (i.e., relatively small territorial males which experience frequent visits by pirates during the nonspawning period) and those territorial males which are unlikely to face sperm competition from pirates (i.e., relatively large territorial males which were not visited by pirates). We predicted that territorial males with piracy risk and sneakers respond to the high expected sperm competition risk by producing higher

quality sperm and have larger relative GM compared with both territorial males with limited piracy risk and pirates themselves. In the second part of this paper, we performed an aquarium experiment on territorial males by exposing some territorial males to a visual stimulus of a pirate nearby (treated) and compare their sperm traits with territorial males without such stimulus (control). We expected treated territorial males to produce higher quality sperm and have larger relative GM compared with the control group.

MATERIALS AND METHODS

Field methods

We observed 14 territorial males for 30 min twice at 4-day intervals (see Ota and Kohda 2006a, 2006b) during November to early December in 2005 at Wonzye Point (lat 8°45.5'S, long 31°06.1'E), near Mpulungu at the southern end of Lake Tanganyika, Zambia using SCUBA diving. Pirates ($n = 7$ in the study area) did not continuously occupy any nests but occasionally intruded into several nests, and territorial males exhibited submissive displays toward them (Ota and Kohda 2006a). Based on these observations, territorial males were divided in those receiving at least 1 visit by a pirate ($n = 8$) and those which did not receive any visit by a pirate ($n = 6$, see RESULTS).

During several days after the last observation, all 14 territorial males and 7 pirates observed were captured, and additionally 42 small individuals (i.e., potential sneakers) were randomly captured from 5 nests. In this study, we did not consider nonparasitic satellite males because we focused on territorial males and parasitic males. All individual males were immediately brought back to the laboratory at Mpulungu and were sacrificed and measured in standard length (SL, to the nearest 0.1 mm using a vernier caliper), body mass (BM, 0.001 g accuracy), and GM (0.001 g) on the day of capture. Of the 42 small individuals, only 14 had white and bulging testes, that is, mature testes that are filled with active sperm (Ota K, Awata S, and Morita M, unpublished data), whereas all territorial males and pirates had active sperm. The testes of each individual male were processed separately: they were carefully removed from the abdomen immediately after sacrificing by cutting the spine at the base of the skull with a sharp pair of scissors. A small amount of milt was directly sampled from a testis using a fine needle and mixed with 15 μ l of lake water on a slide glass. The sperm activity period was measured as the time from mixing to the time when all sperm lost motility (no forward movement) under a light microscope (Nikon, Japan) with a $\times 40$ objective. Each male was measured twice and the average was used. We did not record sperm longevity in replications in which less than 80% of sperm were active immediately after mixing; an additional replicate was conducted in these cases. Repeatability was high ($r = 0.86$, $P < 0.0001$, $n = 35$). Sperm velocity was not measured as we did not have the equipment in Mpulungu to do so.

After measurements of sperm longevity, the unused portion of the testis was fixed in 10% formalin and then flagellum length was measured without prior knowledge of the source males. Parts of the testes were taken from the fixed testes using tweezers, and 20 images were photographed from each testis using a digital camera linked to a light microscope with a $\times 100$ objective. We digitized sperm images on the computer and measured flagellum length using the free software ImageJ 1.34 (available at <http://rsb.info.nih.gov/ij/download.html>). A total of 50–80 spermatozoa were measured for each male (mean \pm standard deviation [SD] = 63.6 ± 8.3 , $n = 33$; excludes $n = 2$ males with missing data, a sneaker and a territorial male: for these 2 males no reliable sperm

measurements could be taken). However, to avoid incorporating sperm with broken flagella in the analyses, we used data on the largest 10 spermatozoa measured only. These 10 spermatozoa were treated as repeated measures of sperm length for a given individual in the analyses (see Data analysis below).

Territorial males from 20 nests near the study site were measured in size, tagged and released to their nests in November 2004, to examine their growth pattern and tactic shift. Five of these males were recaptured on average 347 days later.

Aquarium experiment

The aquarium experiment was conducted in 2008 at the laboratory of Kyoto University, Japan, to test the hypothesis that territorial males can change their sperm traits in response to perceived piracy risk. *Telmatochromis vittatus* captured at Wonye Point were transported to Japan with the permission from Zambian Government. They were kept in storage tanks of 270 l containing many shells on the bottom covered with 2 cm of gravel and coral sand (12:12 h light:dark schedule and water temperature kept between 25–27 °C). Ad libitum, commercial dry food was provided once a day. Each experimental aquarium (60 × 28 × 30 cm) was divided into 2 compartments by a transparent board, one compartment contained a shell as a spawning site (pair compartment) and the other compartment was empty (presentation compartment). First, we introduced a pair ($n = 17$, male 54.3 ± 4.9 mm SL and female 36.7 ± 3.5 mm SL) into the pair compartment (individuals transferred from the storage tanks). After 2 weeks, we either introduced a male (as a visual stimulus of pirate male) larger than the pair-male (62.2 ± 4.4 mm) into the presentation compartment (treated, $n = 8$ pairs) or the presentation compartment was left empty (control, $n = 9$ pairs). Body sizes of pair males did not differ between the treatments: treated males (55.0 ± 1.7 mm SL, $n = 8$) and control males (54.1 ± 1.1 mm, $n = 9$, analysis of variance, $F_{1,15} = 0.25$, $P = 0.63$). The experimental period for each treated and control pair lasted 4 weeks. Note that each pair-female spawned at least once during this period.

After the experimental period, pair-males from both treatments were caught and processed individually as follows: 1) they were sacrificed after anesthetizing using diluted eugenol (FA-100; Tanabe Pharmaceutical Co. Ltd., Japan), 2) BM, GM, and SL were measured, 3) semen was sampled near the genital papilla by fine needles, 4) sampled semen was diluted into 15 μ l of water on a glass slide, 5) sperm quality (longevity and velocity) was determined. Note that in contrast to the field methods (see above), we also measured sperm velocity in this sample (Fitzpatrick et al. 2007). Sperm movements were recorded using a video recorder (GZ-MG555; Victor, Japan) and a CCD camera (63W1N; Mintron, Taiwan) mounted on a microscope (LF-15; Nikon) with a $\times 40$ objective. We recorded sperm movement immediately after the sample was added to water until all visible sperm had stopped moving. The videos were captured at 30 frames/s. Sperm movements were observed frame by frame for consecutive 10 frames (i.e., 0.33 s) at 10, 20, 30, 120, 180, 240, and 300 s after sperm activation. The images were captured for the first 5 frames (i.e., 0.167 s), and the distances that a sperm traveled for the time periods were measured using an image analyzing software package (Image Tracker PTV; Degimo, Co. Ltd., Japan). The mean curvilinear track velocity (VCL) and mean straight-line velocity (VSL) were calculated for 3–43 sperm recorded at each postactivation time period (mean \pm SD = 20.9 ± 7.9 spermatozoa, $n = 136$). We analyzed only spermatozoa whose forward movement was continued throughout the observation of 0.33 s. The sperm longevity was measured as the time since

activation at which 95% of the sperm no longer exhibited progressive motility.

To measure flagellum length, the samples of the 17 males examined were photographed after the video recordings, using a CCD camera (DS-2Mv; Nikon) and an image-processing unit (DS-L2; Nikon) mounted on the microscope with a $\times 100$ objective. Unfortunately, the flagellum lengths of 1 control male and 1 treated male could not be reliably be determined, reducing the sample size from 17 to 15. A total of 16–52 spermatozoa were measured for each male (mean \pm SD = 29.8 ± 10.5 , $n = 15$) using ImageJ 1.34, and mean of the largest 10 sperm were used for the analyses. To avoid observer bias, all samples were measured blind to the treatment.

Data analysis

We tested whether GM allometrically scales with SM (which is BM – GM) and whether this might explain the differences in gonadal investment comparing different males (following Tomkins and Simmons 2002). We calculated residual GM from a common analysis of covariance (ANCOVA) regression slope on SM across tactics to quantify relative gonadal investment.

To examine the effect of male physical conditions on sperm investments, we calculated Fulton's condition factor K : $K = (BM \times 10^5 / SL^3)$ (see Neff and Cargnelli 2004). The 2 measurements of sperm velocity (VCL and VSL) were highly correlated in each postactivation time period (Spearman rank correlation, $r_s > 0.95$, P always < 0.001) and exhibited similar differences depending on the treatments in all analyses (data not shown). Therefore, we show only the results of VCL (see also Liljedal et al. 2008).

In the field data, the males of the 4 different types (i.e., sneakers, types of territorial males, and pirates, see RESULTS) were compared with general linear models (GLMs) (for body size, residual GM, sperm flagellum length, and sperm longevity separately). The models for both sperm quality traits included male body size as a covariate and the interaction between male body size and types. In the aquarium experiment, the males of the 2 treatments were compared with GLM (for residual GM, sperm flagellum length, and sperm longevity separately) or with general linear mixed model (GLMM) (for sperm swimming speed which was repeatedly measured: correcting for the nested effect of male individual identifier within treatment and adding the covariate time since activation). To consider the effects of male body size on VCL, we examined correlations of SL with intercept (i.e., initial VCL) and slope (i.e., decline in VCL) of regression line of each individual male in the GLMM. Trade-offs between sperm traits and male characteristics were analyzed using Pearson's correlations and partial correlations.

To avoid type I errors, we corrected the significant values in multiple comparisons and in multiple but separate correlations within a data set using the Bonferroni method. Normality and homogeneity of variance of the data were checked by Shapiro–Wilks tests and by Levene's tests before analyses, respectively. All analyses were performed by SPSS 17.0.

RESULTS

Field measurements of different male tactics

Pirates did not visit all the 14 nests own by territorial males in the field during the nonspawning period: 8 nests were intruded by pirates (mean frequency = $1.8/\text{h} \pm 1.1$ SD, $n = 8$), whereas 6 remaining nests were never visited during the observations. Subsequently, we divided the 14 territorial males into 2 groups: those with at least one pirate visit ($n = 8$,

Table 1

Differences between the 4 male types in body size and sperm investments in the field ($n = 14$ sneakers, $n = 8$ territorial males with piracy risk, $n = 6$ territorial males with no piracy risk, $n = 7$ pirates; except where indicated due to missing values)

Parameter	Effect	<i>F</i>	df	Error df	<i>P</i>	<i>p</i> polynomial contrast
Body size (SL, mm)	Male type	310.8	3	31	<0.001	<0.001
Testis mass (g)	Male type × SM	2.82	3	31	0.06	—
	Male type	2.59	3	31	0.07	ns
Sperm flagellum length (μm) ^a	Male type × SL	0.53	3	29	0.67	—
	Male type	2.40	3	29	0.09	ns
Sperm longevity (s)	Male type × SL	1.07	3	31	0.38	—
	Male type	4.01	3	31	0.016	<0.02

Results of 4 separate GLMs fitted with a polynomial contrast. ns, not significant.

^a $n = 13$ sneakers and 5 territorial males without piracy risk due to missing values.

“piracy risk”) and those without visits by pirates ($n = 6$). Thus, all adult males were divided into 4 groups, sneakers, territorial males with piracy risk, territorial males without piracy risk, and pirates. Male sizes significantly increased in this group order, and post hoc multiple comparisons showed all 4 types differed except the combination of territorial males without piracy risk and piracy males (Table 1; Figure 1a).

The relationship between GM and soma mass was well explained by a regression line, regardless of the male types (regression: effect of soma mass on GM: $F_{1,33} = 62.8$, $P < 0.001$, $R^2 = 0.65$), and its intercept was highly significantly larger than zero (intercept = 0.006, $F_{1,33} = 1238.7$, $P < 0.001$; Figure 2a). There were no differences in the slopes and intercepts across male types (Table 1). The results suggest that the differences between the male types in GM are ontogenetically determined. Therefore, we simply conclude that relative testis investment follows an ontogenetic trajectory in *T. vittatus*.

Flagellum length did not differ among the 4 male groups (Table 1; Figure 1b) and was not related to body size (linear regression, $F_{1,31} = 2.76$, $P = 0.107$, $R^2 = 0.05$; quadratic regression, $F_{2,30} = 3.09$, $P = 0.061$, $R^2 = 0.12$). Sperm longevity was different among the groups: sneakers and territorial males with piracy risk had longer lived sperm than territorial males without piracy risk and pirates (Table 1; Figure 1c). The differences in sperm longevity depending on the male group could also be described as an effect of body size (Figure 2b): longevity increased with body size in the sneakers and then decreased with body size in the other groups (quadratic regression, $F_{2,32} = 23.85$, $P < 0.001$, $R^2 = 0.57$; quadratic regression gave a significantly better fit than linear regression, $F_{1,33} = 2.76$, $P < 0.001$, $R^2 = 0.34$). Somatic condition neither predicted residual GM (linear regression, $r = -0.25$, $P > 0.1$, $n = 35$), flagellum length ($r = 0.04$, $P > 0.8$, $n = 33$) nor sperm longevity ($r = -0.004$, $P > 0.9$, $n = 35$). There was a tendency of a correlation between sperm longevity and flagellum length ($r = -0.31$, $P = 0.081$, $n = 33$) and no correlation between residual GM and sperm longevity (Pearson's $r = -0.04$, $P = 0.81$, $n = 35$) and between residual GM and flagellum length ($r = 0.18$, $P = 0.32$, $n = 33$; these results did not change when the other variable was controlled for in partial correlations, degrees of freedom [df] = 30: $P = 0.10$, 0.73 and 0.42, respectively).

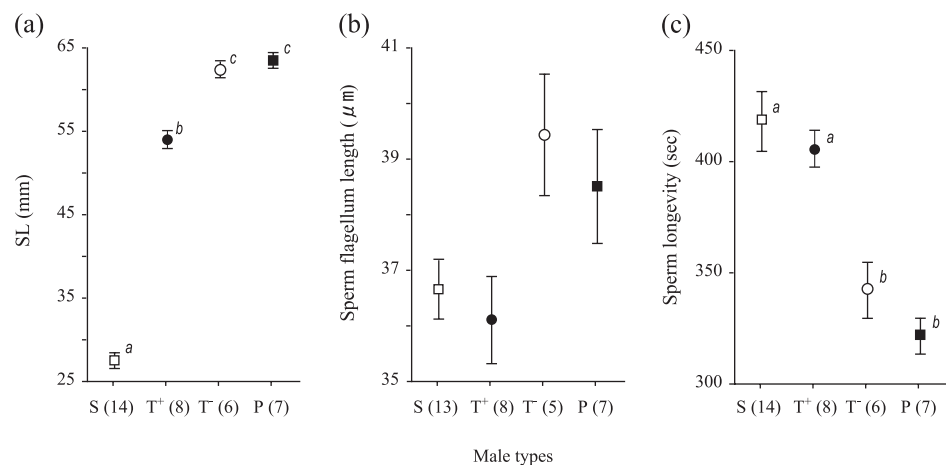
Of the 5 territorial males (mean initial SL was 52.0 mm \pm 3.6 SD in 2004) recaptured 1 year later (2005), one male (63.5 mm) adopted a piracy tactic and the other 4 remained territorial males but were now so large (57.5, 58.0, 60.0, and 62.5 mm) that they no longer were intruded on by pirates (mean frequencies of visits by pirates: 2004: 0.88/h \pm 0.75 SD; 2005: 0.0/h \pm 0.0). Thus, territorial males may outgrow pirates and reduce piracy risk accordingly, and the one shift in tactic is consistent with the idea that only very large territorial males may become pirates.

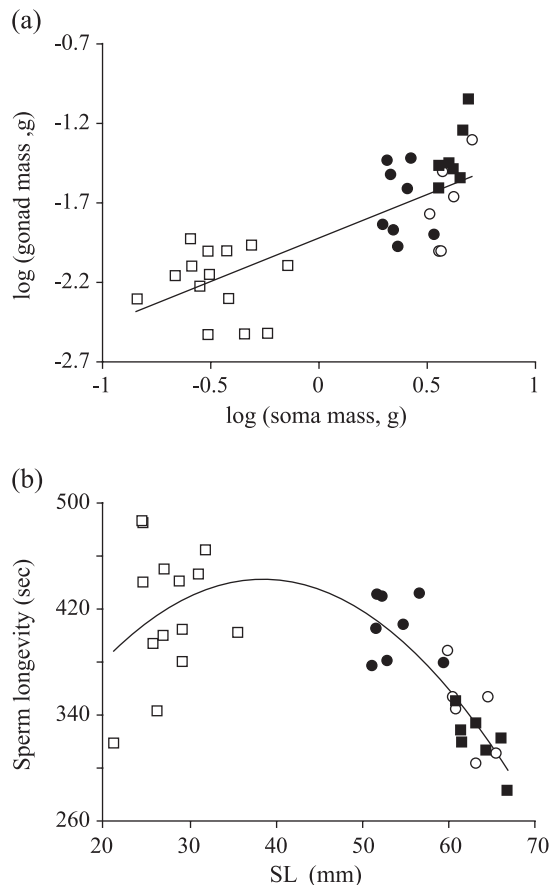
Aquarium experiment of territorial males

In the aquarium experiment, we compared the relative testis investment and the sperm quality of treated males ($n = 8$: with visual contact to a nearby pirate male) with control males ($n = 9$: no visual contact to a nearby pirate male). As in the field situation, testis investment and flagellum length did not

Figure 1

Body sizes and sperm investments of males from the different types in the field (S = sneaker, white squares; T⁺ = territorial with piracy risk, black circles; T⁻ = territorial without piracy risk, white circles; P = pirate, black squares): (a) body size (SL mm), (b) mean of the largest 10 sperm flagellum length (μm), (c) sperm longevity (seconds until all sperm lost forward mobility). Depicted are means \pm standard error of the mean, sample sizes are indicated in the parentheses beside each male group name, and statistical tests are reported in Table 1. Different letters beside the plots denote significant differences.



**Figure 2**

Ontogenetic effects on (a) GM and (b) sperm longevity. (a) An exponential increase of GM through ontogeny in the field (sneaker, white squares; territorial with piracy risk, black circles; territorial without piracy risk, white circles; pirate, black squares). The regression line represents the significant linear relationship. (b) A convex curvilinear relationship between sperm longevity (seconds until all sperm lost forward mobility) and male body size (SL mm) in the field (symbols are same as (a)). The curvilinear regression line represents the significant quadratic relationship ($GM = 175.7 + SM \times (13.81 - 0.178 \times SM)$, see text).

depend on the treatment (Table 2; Figure 3a,b). Again like in the field situation, ANCOVA on log(GM) showed ontogeny was the most important predictor of differences in GM ($n = 17$, effect of covariate log(SM): $F_1 = 6.5$, $P = 0.025$, slope \pm standard error = 1.985 ± 0.868 ; intercept: $F_1 = 119.8$, $P < 0.001$, -2.484 ± 0.325), whereas both treatment ($F_1 = 1.2$, $P = 0.30$) and the interaction treatment \times log(SM) were non-significant ($F_1 = 1.4$, $P = 0.26$). As predicted, treated males had significantly longer lived sperm than control males (Table 2; Figure 3c).

Sperm swimming speed significantly decreased over time since activation (VCL, Figure 3d), but there was no effect of the treatment on sperm swimming speed, which was due to substantial between-individual variation within the same treatment (Table 2). Changing the time effect as a categorical variable with a significant linear, quadratic and polynomial contrast did not affect the treatment significance. However, removing the individual variation and only comparing the means per treatment, treated males had faster swimming sperm in 7 of 8 time periods since activation compared with control males, which is highly significant using a paired t -test

Table 2

Effect of the treatment (control: no pirate $n = 8$; treated: pirate visible $n = 9$) on territorial male sperm investment

Parameter	Effect	<i>F</i>	df	Error df	<i>P</i>	<i>p</i> linear contrast
Testis mass (g)	Treatment \times SM	1.40	1	15	0.26	—
	Treatment	0.17	1	15	0.68	ns
Sperm flagellum length (μm) ^a	Treatment \times SL	0.65	1	13	0.44	—
	Treatment	0.01	1	13	0.92	ns
Sperm longevity (s)	Treatment \times SL	0.47	1	15	0.51	—
	Treatment	8.00	1	15	0.01	0.01
VCL ($\mu\text{m/s}$) ^{b,c}	Treatment \times time	0.01	1	117	0.93	—
	Time	633.2	1	118	<0.001	<0.001
	Treatment	2.20	1	15	0.16	ns

Results of 4 separate GLMs. ns, not significant.

^a $n = 15$ due to missing values.

^b GLMM including the random effect of male individual identifier nested within treatment ($F_{15,118} = 5.77$, $P < 0.001$).

^c See text for the effect of SL on VCL.

($t = -4.92$, $df = 7$, $P = 0.002$). SL was neither correlated with intercept (linear regression, $r = -0.21$, $P > 0.4$, $n = 17$) nor slope of SL-VCL regression ($r = 0.34$, $P > 0.1$, $n = 17$), suggesting that SL has no effects on sperm swimming speed. There were no effects of the magnitude of the size difference between males presented in an aquarium on each sperm quality trait (Spearman rank correlation, residual GM: $r = -0.29$, $P > 0.4$, $n = 8$; flagellum length: $r = 0.29$, $P > 0.5$, $n = 8$; sperm longevity: $r = -0.31$, $P > 0.4$, $n = 8$, $VCL_{\text{intercept}}$: $r = -0.33$, $P > 0.4$, $n = 8$, VCL_{slope} : $r = 0.26$, $P > 0.5$, $n = 8$).

None of residual GM and the sperm quality traits were correlated with body size (Pearson's correlation, residual GM: $r = -0.02$, $P = 0.9$, $n = 17$; flagellum length: $r = -0.12$, $P > 0.7$, $n = 15$; sperm longevity: $r = 0.09$, $P > 0.7$, $n = 17$; $VCL_{\text{intercept}}$: $r = -0.21$, $P > 0.4$, $n = 17$; VCL_{slope} : $r = 0.34$, $P > 0.1$, $n = 17$) and somatic condition (residual GM: $r = 0.05$, $P > 0.8$, $n = 17$; flagellum length: $r = -0.18$, $P > 0.5$, $n = 15$; sperm longevity: $r = -0.28$, $P > 0.2$, $n = 17$; $VCL_{\text{intercept}}$: $r = -0.02$, $P > 0.9$, $n = 17$; VCL_{slope} : $r = -0.20$, $P > 0.4$, $n = 17$) in the experiment. We detected only some pieces of evidence for trade-offs between the sperm quantity and quality traits: significant correlations between relative testis investment and sperm swimming speed measurements (residual GM- $VCL_{\text{intercept}}$: $r = -0.59$, $P = 0.012$, $n = 17$, residual GM- VCL_{slope} : $r = -0.72$, $P = 0.001$, $n = 17$) and between sperm swimming speed measurements ($VCL_{\text{intercept}}$ - VCL_{slope} : $r = -0.89$, $P < 0.0001$, $n = 17$), although the former correlations were not significant when the significant level was adjusted. Thus, sperm with faster initial swimming speed would decline more rapidly the speed. Otherwise correlations were nonsignificant (residual GM-flagellum: $r = -0.03$, $P = 0.91$, $n = 15$; residual GM-longevity: $r = 0.33$, $P = 0.20$, $n = 17$; flagellum-longevity: $r = -0.15$, $P = 0.59$, $n = 15$; flagellum- $VCL_{\text{intercept}}$: $r = 0.02$, $P > 0.9$, $n = 15$; flagellum- VCL_{slope} : $r = -0.26$, $P > 0.3$, $n = 15$; longevity- $VCL_{\text{intercept}}$: $r = 0.15$, $P > 0.5$, $n = 17$; longevity- VCL_{slope} : $r = 0.23$, $P > 0.3$, $n = 17$). The 3 nonsignificant correlations between sperm traits did not change when corrected for residual GM in partial correlations (all 3 $P > 0.25$).

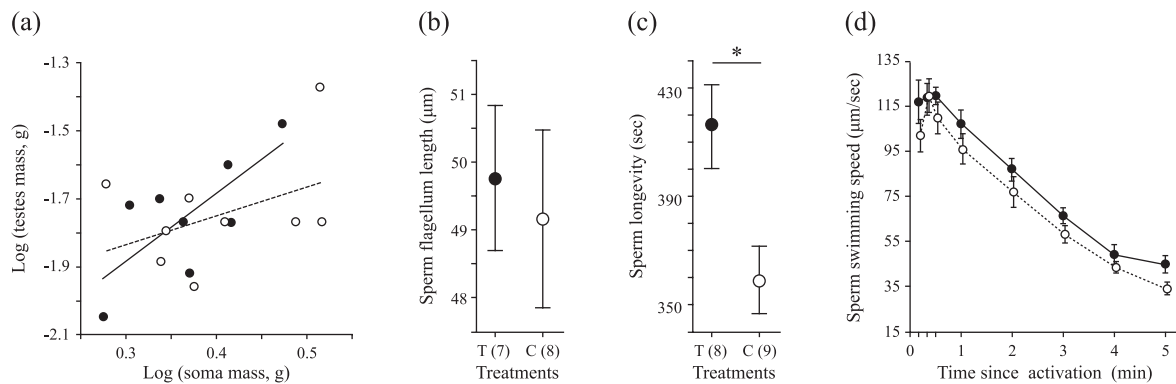


Figure 3

Sperm investments of paired males from the 2 different treatments in the laboratory (C = control males, white circles; T = treated males, i.e., exposed to a pirate behind glass, black circles): (a) relative gonad investment, (b) mean of the largest 10 sperm flagellum length, (c) sperm longevity (seconds until all sperm lost forward mobility), (d) sperm swimming speed since time of activation (VCL, $n_{\text{control}} = 8$ and $n_{\text{treated}} = 9$). Depicted are means \pm standard error of the mean, sample sizes are indicated in the parentheses beside each treatment, and statistical tests are reported in Table 2. An asterisk denotes a significant difference between the treatments.

DISCUSSION

Our field observations confirmed that only small territorial males faced the risk of spawning takeover by pirates: pirates visited nests of these males in the nonspawning period, and thereby presumably verified whether the female was ready to spawn. In contrast, large territorial males were very similar in size to the pirates and consequently they may be able to deter pirates from their nests (and perhaps choose nest sites with lower piracy risk, Ota and Kohda 2006b; submitted) and were never visited by any pirate. Consequently, small territorial males visited by pirates appeared to adjust to the risk of facing sperm competition during spawning with a pirate by producing longer lived sperm with shorter flagella compared with the large territorial males whose nests were not visited by pirates. Male body size appeared a good proxy of the ART performed by the male. Results of the aquarium experiment were partly consistent with the field observations: already visual exposure to a potential pirate was sufficient to induce territorial males to produce longer lived sperm (again no adjustments were visible in relative testes mass and in sperm flagellum length) compared with control males which were not exposed to a pirate. As in the field situation, relative testes mass was mainly depending on male body size independent of the treatments.

Sperm from treated pair males swam significantly faster than sperm from control pair males only after removing the substantial between-individual variation in sperm swimming speeds. This suggests that other factors, which appear to be male individual specific, affected sperm swimming speeds much more strongly than our treatment. The size difference between males was not the explanation for the individual effect. Therefore, we recommend to compare any treatment likely to affect male sperm investment in future experiments in a repeated measures design (i.e., each male receives both control and treatment in randomized order) and additionally search for other factors explaining between-individual variations in sperm swimming speeds, which are not controlled or considered in the experiment (e.g., shell size, the direct contacts between males, and the presence of sneakers).

Moreover, all nests of territorial males contained up to 16 very small males (Ota K, unpublished data), 33% ($n = 42$) of which had functioning testes and could potentially sneak fertilizations during spawning ("sneakers," Ota and Kohda 2006a). Sneakers produced sperm with the highest longevity of any male, whereas pirates produced sperm with shortest

longevity of any males. This difference is probably due to sneakers always facing sperm competition with at least 1 larger male (either a territorial male, a pirate, or both), whereas pirates only rarely face sperm competition with another male because during spawning they usually can deter all territorial males and sneakers from the spawning site (but occasional sneaking by both territorial males and sneakers does occur; Ota 2007). Unfortunately, no sperm swimming speed measurements could be made in the field to verify whether they also produced faster swimming sperm. Taken together, the increase in sperm longevity (and maybe also sperm swimming speed: needs to be tested in the future in sneakers and pirates) closely matches the expected likelihood of facing sperm competition in male *T. vittatus*: sneakers = small territorial with pirate visits > large territorial males without pirate visits = pirates. Our results compare well to another study performed in the wild *T. vittatus* population (Fitzpatrick et al. 2007: performed in the Kasakalawe population 8 km from our study population at Wonzye Point). They examined sperm quality traits (which included swimming speed) depending on male tactics and reported no differences in sperm morphology among tactics, whereas sperm longevity increased from sneakers = territorial males > pirates (but not significant due to the small sample sizes and very high variability in both territorial males and pirates) and sperm swimming speed increased from sneaker > territorial males > pirates (significant, with notably sneaker males retaining faster and more straight swimming sperm 6 min after activation, see Fitzpatrick et al. 2007 for details). Similarly, large investments in sperm quality traits by small sneakers in disfavored role have been documented in other fish species (e.g., Uglem et al. 2001; Vladoić and Järvi 2001; Neff et al. 2003; Burness et al. 2004; Rudolfsen et al. 2006; Locatello et al. 2007).

Territorial male *T. vittatus* are likely to experience ontogenetic changes in the risks of sperm competition. The limited long-term field observations showed that small territorial males grow to large territorial males and finally to pirates, indicating that these tactics are not fixed for life. Male *T. vittatus* were freed from nest piracy as they grow, suggesting that territorial males increase reproductive success as growing. Hence, it is most likely that male ARTs in *T. vittatus* are explained by the status-dependent selection model (Gross 1996). Our experiment shows that if territorial males face piracy risk, their sperm longevity and sperm swimming speed will increase. Taken together, territorial male *T. vittatus* seem

to plastically change sperm quality in response to the size-dependent likelihood of nest piracy.

Why do *T. vittatus* males facing higher sperm competition risk produce longer lived sperm than males facing lower risk? Females of this fish sequentially deposit single eggs over a prolonged time period of more than 3 h inside their shells. It is likely that sneakers, and territorial males faced with a pirate takeover, cannot time their sperm release closely to each egg being laid, so sperm remaining viable for a longer time period will be much more likely to fertilize at least some eggs. Note that male ejaculates into the shell, so sperm will not quickly dilute or diffuse from the shell and may remain sufficiently long viable to fertilize subsequent eggs being laid (Scaggiante et al. 1999; Locatello et al. 2007). In this situation, longer lived sperm may have a better chance of fertilizing eggs, particularly when males ejaculate directly before a female lays her eggs, as Fitzpatrick et al. (2007) previously argued (see Kanoh 1996; Reichard et al. 2004; Locatello et al. 2007 for examples of other externally fertilizing fish). Thus, longer lived sperm will be advantageous for both sneakers and also for the small territorial males that employ sneaking as counterstrategy against pirate male takeovers.

We should consider alternative hypotheses that might explain the variation in sperm longevity among different males of *T. vittatus*. Of the sperm traits examined in this study, only longevity and speed were the changeable traits and appeared not to be traded-off with other sperm quantity and quality traits, suggesting that the variation in longevity and speed may not be a by-product of adjustments in sperm numbers and morphology. The only exception was an apparent trade-off between relative testes mass and maximum sperm swimming speed found in the laboratory (where the maximum is usually attained directly after sperm activation). Production of higher quality sperm will demand more energy from the males and might only be possible for males in better body condition (Urbach et al. 2007; Burness et al. 2008). However, we detected no correlation between sperm longevity and male body condition. Furthermore, sperm might deteriorate in quality with male age (Urbach et al. 2007; Rudolfsen, Muller, et al. 2008; Pizzari et al. 2008), which can be approximated with male body size in our data because fish show indeterminate growth. In our aquarium experiment, however, control and treated territorial males were matched in size, so age/size-dependent changes in sperm quality traits cannot explain our experimental results. Nevertheless, sneaker males increased the longevity of their sperm with their size (as indicated by the significant quadratic relationship) and because size appears to be a close proxy of the role males are likely to play and their relative testes mass (male type: sneaker, territorial, or pirate), age/size effects can never be easily disentangled from male type effects. For instance, maybe larger sized sneakers are more likely to actually perform sneaking or perform sneaking more often than smaller sized sneakers, which might explain the quadratic relationship mentioned above. Taken together, the observed within-tactic and between-tactic variations in sperm longevity (and swimming speed) and relative testes mass link to the sperm competition risks that each male experiences, which in turn is mostly affected by the male's (relative) body size compared with the other males in the population.

Finally, we have assumed that all males release their sperm at the shell entrance. This is not exactly true. Very small sneaker males may wriggle past the spawning female into the deepest part of the shell and release their sperm there very close to where the eggs are actually deposited. In contrast, larger sized sneakers might not pass the female and have to release their sperm at the entrance, similar to the territorial males and pirates. These body size-dependent sneaker tactics closely ap-

proximates the body size-dependent dwarf male tactics in the closely related cichlid *L. callipterus*: large dwarfs cannot enter the shell (particularly if the shell and the female are small) and employ dashing sneaking over the entrance of the shells, whereas small dwarfs wriggle past the female and sneak inside the shell (Sato et al. 2004; Schütz et al. 2010). Therefore, in *T. vittatus* only small sneakers will succeed in wriggling sneaking and they will experience a decrease in the possibility of successful wriggling as they grow. Consequently, they are expected to produce higher quality sperm as they grow to counterbalance this disadvantage, exactly as is apparent in Figure 2b. Whether sneakers are able to change their sperm quality traits according to the likelihood of performing wriggling or dashing sneaking tactics will be experimentally tested in the near future.

To our knowledge, this study provides the first evidence that sperm competition risks are variable within a tactic of a population of a single species, where even visual exposure of a potential competitor suffices to induce the effect. We further suggested that male *T. vittatus* ontogenetically change sperm quality traits as body size largely determines ARTs employed and accordingly determines the likelihood of facing sperm competition from smaller and/or larger males in the population. Our experimental results imply that sperm traits can be adjusted plastically on a short-term basis and therefore corroborates other recent findings on sperm phenotypic plasticity (e.g., Rudolfsen et al. 2006; Cornwallis and Birkhead 2007, 2008; Pizzari et al. 2007; Simmons et al. 2007; Ramm and Stockley 2009).

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